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ORIGINAL ARTICLE

# Separation and purification and in vitro anti-proliferative activity of leukemia cell K562 of *Galium aparine* L. petroleum ether phase



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## KEYWORDS

*Galium aparine* L.;  
Purification;  
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**Abstract** To explore material basis of in vitro anti-proliferative activity of leukemia cell K562 of petroleum ether phase of product resulting from *Galium aparine* L. 60% ethanol extraction, the experiment adopts column chromatography combined with thin layer preparation, isolates and purifies petroleum ether, conducts structural identification of obtained single compound and applies MTT method for viability assay of in vitro anti-proliferative activity of leukemia cell K562. Experimental results show that *G. aparine* L. petroleum ether contains mainly  $\beta$ -sitosterol, daucosterol and dibutyl phthalate and other substances. Under experimental conditions, the three could inhibit the proliferation of leukemia cell K562 with dose-effect and time-effect relationship, of which dibutyl phthalate has strongest activity. Dibutyl phthalate with excellent activity,  $\beta$ -sitosterol with rich content and moderate effect should be the main contributor to its biological activity.

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## 1. Introduction

*Galium aparine* L. is a common weed in wheat and barleyfields, also known as *Rubia cordifolia*, *Scutellaria tuberifera*, *G. aparine*, catchweed bedstraw herb. As herbaceous plant of gallium

of rubiaceae, it was first recorded in *Yunnan Materia* as Chinese herbal medicine. Traditional medicine holds that *G. aparine* L. can clear away damp-heat, eliminate stasis, dissipate detumescence and detoxify, which can be used for treatment of stranguria with turbid urine, hematuria, traumatic injury, acute appendicitis, furuncle, otitis media, etc. In modern clinical medicine, it is also used for treatment of cancer, especially leukemia (Yang and Yang, 1975). Among these traditional Chinese medicine prescription and Chinese medicinal formulae for treatment of tumors, *G. aparine* L. serves as principal drug, which indicates that it has ingredients to eliminate evil and can suppress tumor cells. However, its material basis for antitumor activity has not been mentioned

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in current domestic and international research (He and Zheng, 1994; Health Center in Sandian District, 1972; Chen, 2002; Peng et al., 2015; Wang, 2005; Zhao and Zhao, 2006; Li and Chen, 2004; Ling, 2009; Bojko, 1995; Kirillov et al., 2002).

We once adopted ethanol of different concentrations to extract catchweed bedstraw herb. Activity tracking results show that petroleum ether phase of material extracted by 60% ethanol can well inhibit leukemia cell viability. In this paper, column chromatography combined with thin layer preparation and recrystallization method is adopted to separate monomer compound, and in vitro anti-proliferative activity of leukemia cell K562 detection of the resultant monomer compound is done in order to find antitumor activity substances and lay a foundation for further development and utilization.

## 2. Experimental method

### 2.1. Materials, equipment and reagents

*G. aparine* L. whole plant which was collected from Maozhong, Zhengzhou, in May 2009, and identified by Associate Professor Yang Huaixia of Henan University of Traditional Chinese Medicine as herb *G. aparine* L. of *G. aparine* of rubiaceae.

Flash EA1112 element analyzer from US Thermo Electron SPA company; Nexus470 intelligent Fourier transform infrared spectrometer from US Nicolet company; Avance-300 superconducting NMR spectrometer, Avance-400 superconducting NMR spectrometer, from Germany Bruker; Agilent 1100 LC-MSD-Trip-XCT, from US Agilent company; carbon dioxide incubator from Shanghai Yiheng company; clean bench from Suzhou purification equipment plant; BIO-RAD680 microplate reader from US Bio-Rad company; micro-injector from Germany eppendorf company; high-speed centrifuge from US sigma company; culture plate from US costar company.

MTT from US merck; RPMI1640 medium from Gibco Company; fetal bovine serum from Chinese Academy of Medical Sciences; other reagents are domestic analytical pure.

### 2.2. Methods

#### 2.2.1. Extraction

Weigh 200 g dried crushed *G. aparine* L. whole plant, conduct 2 h reflux extraction of 1000 mL of 60% ethanol, extract twice, filtrate with vacuum concentration and obtain extract, 3 kg materials are used and 624.5 g extract is obtained.

#### 2.2.2. Extraction

Disperse extract with appropriate amount of distilled water, extract with petroleum ether, conduct vacuum concentration, evaporate petroleum ether and obtain 20.8 g petroleum ether phase.

#### 2.2.3. Preparation

For obtained petroleum ether phase, use petroleum ether: ethyl acetate as eluent for column chromatography, conduct gradient elution, test sample with thin layer analysis board, conduct color detection with UV fluorescence combined with sulfuric acid – methanol and acetic anhydride – concentrated sulfuric acid (Liebermann–Burchard reaction); for eluted components,

use petroleum ether: ethyl acetate, petroleum ether: chloroform and chloroform: methanol as the developing solvents, repeatedly prepared with silica gel GF254 preparative thin layer, 3 single compounds are obtained, with concrete results shown in Table 1.

### 2.2.4. Anticancer activity assay (MTT method)

Formulate different monomer compounds into DMSO solution with concentration at 0.1 mol/L, draw 10  $\mu$ L and dilute with medium to 1 mL and obtain liquid to be measured. Take K562 cells of logarithmic growth phase to be inoculated in three 96-well plates with density at  $1 \times 10^5/\text{cm}^2$ , each well with 100  $\mu$ L. Add 4, 8, 12, 16, and 20  $\mu$ L soup into each well of sample set, and supplement volume to 200  $\mu$ L with medium. Blank group (only added with culture solution) and control group (without drug) are set, with six parallel for each group. After cultured for 48 h, 36 h and 72 h, add MTT, culture for 4 h, centrifuge, discard supernatant, and add 150  $\mu$ L DMSO to each well to fully dissolve the crystals. Measure absorbance at 570 nm wavelength with microplate reader (Bio-Rad 3350 microplate reader). With blank zero, perform parallel experiments twice and take average value. Calculate cell viability with the following formula:

$$\text{cell viability} = (\text{absorbance of experimental group} / \text{absorbance of control group}) \times 100\%.$$

## 3. Results and analysis

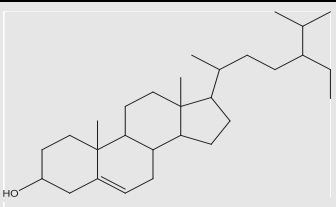
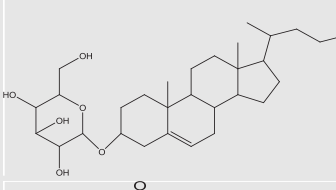
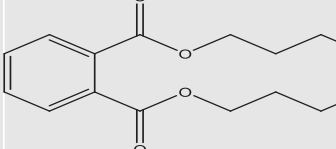
### 3.1. Structural analysis of compound

#### 3.1.1. Compound I ( $\beta$ -sitosterol)

As white powder solid, it is positive in sulfuric acid – methanol and acetic anhydride – concentrated sulfuric acid reaction, and negative in Molish reaction, which indicates that the compound is a steroid or triterpenoids aglycone; ESI-MS shows molecular ion peak  $m/z$ : 413  $[\text{MH}]^+$ , elemental analysis result: C element content is 83.92%, H elemental content is 12.06%. Combined with NMR data, compound formula is speculated as  $\text{C}_{29}\text{H}_{50}\text{O}$ . Calculation obtains that degree of unsaturation is 5, which indicates that there may be double bond and ring in compound structure. In infrared IR ( $\text{cm}^{-1}$ ) spectrum: stretching vibration absorption peak at  $3425\text{ cm}^{-1}$  shows the presence of hydroxyl, methyl signal at  $2960\text{ cm}^{-1}$  and  $2866\text{ cm}^{-1}$ , and methylene signal at  $2935\text{ cm}^{-1}$  and  $2851\text{ cm}^{-1}$ ; absorption peak relatively weak at  $1641$  confirms the presence of double bond.

Nuclear magnetic resonance hydrogen spectrum data indicate the presence of continuous peak envelope at  $\delta 2.27\text{--}1.03$ , which may be because of steroid nucleus backbone and side-chain hydrogen information generated due to overlap of numerous methylene and sub-methylene signal on steroid skeleton.  $\delta 5.34$  and  $\delta 3.51$  prove the existence of a unsaturated CH and hydrogen on one carbon monoxide; nuclear magnetic resonance spectroscopy only shows two C chemical shift information in unsaturated carbon atom region (except alkyne carbon atoms):  $140.7\text{ ppm}$ ,  $121.7\text{ ppm}$ , which should be ene carbon signal; C NMR data show a total of 29 carbon in compounds. According to dept, it can be seen that there are a total of 6-methyl, 3 quaternary carbon, 11 methylene, 9 methine.

**Table 1** Compound contained in petroleum ether phase of *Galium aparine* L.

Category	No.	Name	Structural formula
Steroid	Compound I	$\beta$ -sitosterol	
	Compound II	Daucosterol	
Other	Compound III	Dibutyl phthalate	

Specific NMR data are as follows:

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 5.34 (1H, t,  $J = 5.2$  Hz, H-6), 3.51 (1H, m, H-3), 0.68 (3H, s,  $\text{CH}_3$ -18).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$ : 140.7 (C-5), 121.7 (C-6), 71.8 (C-3), 56.8 (C-14), 56.1 (C-17), 50.2 (C-9), 45.8 (C-24), 42.3 (C-13), 42.2 (C-4), 39.8 (C-12), 37.3 (C-1), 36.5 (C-10), 36.2 (C-20), 33.9 (C-22), 31.9 (C-7), 31.9 (C-8), 31.6 (C-2), 29.1 (C-25), 28.3 (C-16), 26.1 (C-23), 24.3 (C-15), 23.1 (C-28), 21.1 (C-11), 19.5 (C-19), 19.4 (C-26), 19.1 (C-27), 18.9 (C-21), 12.0 (C-29), 11.8 (C-18). After consulting the literature, it is found that the aforesaid data are basically consistent with  $\beta$ -sitosterol data reported in the literature (Ewa et al., 2013). Therefore, compound I is identified as  $\beta$ -sitosterol.

### 3.1.2. Compound II (daucosterol)

As white powder solid, it is positive in Molish reaction and shown fuchsia after heated in 10% sulfuric acid – methanol solution. ESI-MS shows molecular ion peak  $m/z$ : 575  $[\text{MH}]^+$ , elemental analyzer test results: C element content is 72.83%, H element content is 10.39%. Combined with NMR data, it is speculated that the compound formula is  $\text{C}_{35}\text{H}_{60}\text{O}_6$ . Calculation obtains that degree of unsaturation is 6, which indicates that the compound structure may contain double bond and ring. In infrared IR ( $\text{cm}^{-1}$ ) spectrum: stretching vibration absorption peak at  $3416\text{ cm}^{-1}$  shows the presence of hydroxyl, methyl signal at  $2958\text{ cm}^{-1}$  and  $2869\text{ cm}^{-1}$ , methylene signal at  $2933\text{ cm}^{-1}$ ; relatively weak absorption peak at  $1627\text{ cm}^{-1}$  confirms the presence of double bond.  $1255\text{ cm}^{-1}$  represents characteristic absorption peak of C–O stretching vibration in ether bond, while  $1075\text{ cm}^{-1}$ ,  $1025\text{ cm}^{-1}$  serve as acetal characteristic signal of pyranose.

Nuclear magnetic resonance hydrogen spectrum shows  $\delta 4.11$ – $2.14$  continuous peak envelope, which may be numerous methyl, methylene and sub-methylene signals on steroid skeleton and side chain. Nuclear magnetic resonance spectroscopy shows 35 carbon signals, with three C chemical shift information for unsaturated carbon atom region (except alkyne carbon atoms): 142.2 ppm, 123.2 ppm and 103.6 ppm, of which

142.2 ppm, 123.2 ppm should be ene carbon signal, 103.6 ppm should be sugar anomeric carbon signal; 63.9–79.5 are sugar oxygen containing substituted aliphatic carbon signal. Except sugar carbon signal, signal of nuclear magnetic resonance spectroscopy of compound II is very close to that of compound I.

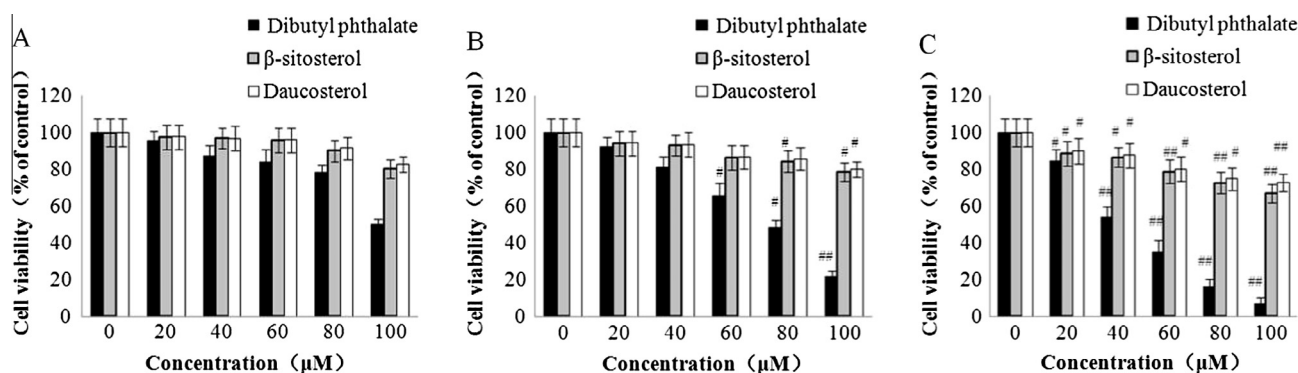
Specific NMR data are as follows:

$^{13}\text{C}$  NMR (100 MHz,  $\text{Pyr-d}_5$ )  $\delta$ : 142.2 (C-5), 123.2 (C-6), 103.6 (C-1'), 79.6 (C-3), 79.5 (C-3'), 79.4 (C-5'), 76.3 (C-2'), 72.8 (C-4'), 63.9 (C-6'), 58.1 (C-14), 57.5 (C-17), 51.6 (C-9), 47.3 (C-24), 43.8 (C-13), 40.5 (C-12), 38.8 (C-4), 38.2 (C-1), 37.6 (C-10), 35.5 (C-20), 33.4 (C-22), 33.3 (C-7), 31.4 (C-8), 31.3 (C-2), 30.7 (C-25), 29.8 (C-16), 27.6 (C-23), 25.8 (C-15), 24.6 (C-28), 22.5 (C-11), 21.3 (C-27), 20.8 (C-19), 20.5 (C-26), 20.3 (C-21), 13.2 (C-29), 13.2 (C-18). After consulting the literature, it is found that the aforesaid data are basically consistent with daucosterol data reported in the literature (Mohammad and Mahdi, 2014). Therefore, compound II is identified as daucosterol.

### 3.1.3. Compound III (dibutyl phthalate)

In light yellow oil, it shows molecular ion peak  $m/z$  in ESI-MS: 279  $[\text{M} + \text{H}]^+$ , 301  $[\text{M} + \text{Na}]^+$ , elemental analyzer test results: C element content is 68.96%, H element content is 7.89%, and thus the compound formula is  $\text{C}_{16}\text{H}_{22}\text{O}_4$ . Calculation obtains that degree of unsaturation is 6, which indicates that the compound structure may contain benzene and two double bonds or ring. In infrared IR ( $\text{cm}^{-1}$ ) spectrum: 2960 and  $2874\text{ cm}^{-1}$  are methyl signal,  $2934\text{ cm}^{-1}$  is methylene signal;  $1728\text{ cm}^{-1}$  is carbonyl absorption, 1600, 1580, and  $1466\text{ cm}^{-1}$  are benzene ring skeleton vibration.

Nuclear magnetic resonance hydrogen spectrum  $\delta 0.91$  shows two methyl of symmetric structure,  $\delta 1.44$ ,  $\delta 1.72$  show four symmetrical methylene,  $\delta 4.31$  shows two symmetrical oxygen methylene; C NMR at 132.3 ppm, 130.9 ppm, 128.8 ppm show aromatic carbon information, 167.7 ppm shows the presence of carboxylic acid. Specific NMR data are as follows:



**Figure 1** Cytotoxicity of dibutyl phthalate,  $\beta$ -sitosterol and daucosterol on K562 cells (K562 cells were treated with dibutyl phthalate,  $\beta$ -sitosterol and daucosterol at various concentrations for 24 h (A), 48 h (B), 72 h (C) and cell viability was determined by MTT assay. Data were shown as mean  $\pm$  SD ( $n = 6$ ).  $\#p < 0.05$ ,  $\#\#p < 0.01$  compared with the control).

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.72 (1H, dd,  $J = 3.3$ , 3.5 Hz, H-3, H-6), 7.53 (1H, dd,  $J = 3.3$ , 3.3 Hz, H-4, H-5), 4.31 (4H, t,  $J = 6.3$  Hz, H-8, H-8'), 1.72 (4H, m,  $J = 6.3$  Hz, H-9, H-9'), 1.44 (4H, m,  $J = 6.8$  Hz, H-10, H-10'), 0.91 (6H, t,  $J = 7.3$  Hz, H-11, H-11');  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$ : 167.7 (C-7, 7'), 132.3 (C-1, 2), 130.9 (C-4, 5), 128.8 (C-3, 6), 65.5 (C-8, 8'), 30.6 (C-9, 9'), 19.2 (C-10, 10'), 13.7 (C-11, 11'). After consulting the literature, it is found that the afore-said spectral data are basically consistent with dibutyl phthalate data reported in the literature (Zhang et al., 2006). Therefore, the compound is identified as dibutyl phthalate.

### 3.2. Assay of in vitro anti-proliferative activity of leukemia cell K562

By column chromatography combined with thin layer preparation, isolate and identify three single compounds from petroleum ether phase extracted from 20.8 g catchweed bedstraw herb with 60% ethanol: 6.3 g  $\beta$ -sitosterol, 281 mg daucosterol and 962 mg phthalate butyl. Test results of in vitro anti-proliferative activity of K562 cell are shown in Fig. 1.

As can be seen from Fig. 1, under condition of experimental concentration, the three can inhibit the proliferation of leukemia cell K562, wherein  $\beta$ -sitosterol and daucosterol have moderate inhibition effect, while dibutyl phthalate has very obvious effect. Their inhibition ability shows dose-effect and time-effect relationship. That is, with increase in dose and extension of dosing time, inhibition effect gets stronger.

## 4. Conclusion

Three single compounds were separated from 60% ethanol petroleum ether phase of *G. aparine* L., respectively  $\beta$ -sitosterol, daucosterol and dibutyl phthalate. Dibutyl phthalate has been found in many plants. For instance, Zhang Donghui obtained such substance from *Stellera chamaejasme*, Diao Mingyuan from *evodia lepta* of rutaceous plant, Li Xiaobao from roots of *Polyalthia laui* in Hainan. Studies by Chu-tse Wu et al. have shown that this compound can significantly kill leukemia cells in vivo and in vitro selectively, and its mechanism may be related to dibutyl phthalate characteristic that it can affect expression of some oncogenes and tumor suppressor genes and thus induce tumor cell apoptosis (Wu et al., 1993). Our experimental results also show that dibutyl

phthalate has excellent in vitro anti-proliferative activity of leukemia cell K562.

Dibutyl phthalate with excellent activity and  $\beta$ -sitosterol with rich contents and moderate anti-tumor activity should be material basis for catchweed bedstraw herb with 60% ethanol to extract biological activity from petroleum ether phase.

## References

- Bojko, I.A., 1995. A method for treating tumor. Russ. 2028808.
- Chen, F.Z., 2002. One preparation method of "Blood cancer powder". China: 1339304A.
- Ewa, S.S., Roman, P., Paul, H., et al, 2013. Characterisation of non-polar dimers formed during thermo-oxidative degradation of  $\beta$ -sitosterol. Food Chem. 139, 464–474.
- He, Z.J., Zheng, X.C., 1994. Report of treatment of three cases of acute leukemia with survival of more than nine years mainly by supplementing Qi and nourishing Yin. Shanghai J. Traditional Chin. Med. 11, 24–25.
- Health Center in Sandian District, Xinzhou County, Hubei Province, 1972. Preliminary clinical report of treatment of 15 cases of tumor with Bedstraw. New Medicine, 9, pp. 26–27.
- Kirillov, N.A., Mitrasov, Y.N., Ionova, E.A., et al., 2002. A medicated liquor containing medicinal composition for treating and preventing diseases. Russ. 2192877.
- Li, S.Z., Chen J.M., 2004. A drug for treatment of lung cancer. China: 1530133A.
- Ling, Z.X., 2009. A decoction medicine for treatment of rectal cancer hemafecia. China: 1733008A.
- Mohammad, A.E., Mahdi, M.F., 2014. Inactivation of PI3K/Akt pathway and upregulation of PTEN gene are involved in daucosterol, isolated from *Salvia sahendica*, induced apoptosis in human breast adenocarcinoma cells. S. Afr. J. Bot. 93, 37–47.
- Peng, W.X., Wang, L.S., Makoto, O., Zhang, M.L., 2015. Separation of hemicelluloses from *Eucalyptus* species: investigating the residue after alkaline treatment. Cellul. Chem. Technol. 49, 756–764.
- Wang, D.L., 2005. Chinese herbal preparation for treatment of Leukemia. China: 1695703A.
- Wu, C.T., Jiang, Y.M., Cao, J.R., et al, 1993. A new pharmacological function of phthalate ester plasticizer. Leuk. Res. 11, 1013–1016.
- Yang, Y.J., Yang, Y.X., 1975. Upper stony flat abscess. Acta Universitatis Medicinalis Anhui 1, 18.
- Zhang, L.T., Sun, J.L., Gu, X.B., et al, 2006. Separation and purification and structural identification of secondary metabolites of actinomycetes SPRI0518. Chem. Bioeng. 9, 29–33.
- Zhao, S.Z., Zhao C.G., 2006. Chinese medicine composition for treatment of Leukemia. China: 1733008A.